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Some Observations on Angular Leaf Spot of Cucumber with a Scanning Electron Microscope

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Pseudomonas lachrymans, the causal bacterium of angular leaf spot of cucumber, was inoculated by a pressure spraying method with a spray gun on the leaves of cucumber plants and the surface structure of the lesions formed on inoculated leaves was observed with a scanning electron microscope. No wounds were produced on the leaf surface inoculated by this method. Many bacteria were found in the stomatal pores immediately after inoculation. Therefore, the bacterial cells might enter through natural openings, i.e. stomata. One to two days after inoculation a large number of bacteria which multiplied in the tissue egressed from the stomata. These bacteria may be available for secondary infections. During the 3 days after inoculation most of the cells within the lesion gradually lost their turgor pressure and thereafter became completely flat. The lesions were concave and sharply distinguished from the surrounding areas. Many small cracks were produced on the older lesions, probably due to marked dryness of the tissue within the lesions. Many bacteria were observed in these cracks. These bacteria also may be useful for new infections.

It is reported that the ingress into the plant tissues by plant pathogenic bacteria occurs through natural pores. Stomata are obviously excellent parts of entry for bacteria, although bacteria are capable of entering through various natural pores in the cuticles. In this experiment, we used the method by which bacteria are forcibly poured into the host tissues through the stomata only. By using this method, the inoculated bacteria can enter through the stomata immediately after inoculation.

This paper describes the results of some observations on stomatal invasion and lesion formation by *Pseudomonas lahrymans*, the causal bacterium of angular leaf spot of cucumber, using a scanning electron microscope.

Materials and Methods

The cucumber cultivar (Cucumis sativus L.) used in this experiment was Yamatosanjyaku, which is susceptible to angular leaf spot. Plants were grown in 5-inch pots in a glasshouse and used at the 3rd leaf stage.

An isolate No. 7417 of *P. lachrymans* used in this experiment was kindly supplied by Mr. T. Kimura, Miyagi Agricultural Junior College, Natori. The isolate was grown on Wakimoto's medium (15 g sucrose, 5 g peptone, 0.5 g calcium nitrate, 2 g sodium phosphate, dibasic, and the broth from 300 g of boiled potatoes made to 1 liter, pH 7.0) for 48 hr at 27°C. To prepare the inoculum, bacteria were washed from agar slants with distilled water, centrifuged, resuspended in fresh distilled water and adjusted photometrically to an absorbance of 0.1 at 620 nm (ca. 10^8 cells/ml)

For the inoculation the method of Omer and Wood (1) was used with a slight modification as follows. The bacterial suspension was applied to the selected circular parts, 5 mm in diameter, on the under-surface of the leaves through the fine nozzle of an artist spray gun at a pressure of 20 lb from a distance of 1 cm for 4–6 sec. Inoculated plants were kept in a moist chamber held at 25°C for 48 hr, after which they were returned to the vinyl-box in the glasshouse.

The specimens for the observation with a scanning electron microscope were taken at 0, 1, 2, 3, 4 and 8 days after inoculation and then they were prefixed for 2 hr with 2.5 per cent glutaraldehyde buffered with 0.2 M phosphate buffer at pH 7.2. After washing out the fixative with the buffer, the material was postfixed in 2 per cent osmic acid in the same buffer for 1 hr, rinsing in 0.85 per cent saline solution. The specimens were slowly dehydrated with an alcohol series and inpregnated with n-amyl acetate, which were next dried by the critical point dry method. Each fragment was mounted on a galvanized metal stub and plated with gold to make the surface electrically conductive. The specimens were observed with a scanning electron microscope Hitachi S-700 at 30° to the beam and with accelating voltage of 15 kv.

Results and Discussion

The circular parts of cucumber leaves inoculated by spraying forcibly bacterial suspension with a spray gun, were temporarily water-soaked, but the water-soaking disappeared within 2 hr. One day after inoculation many water-soaked spots appeared on the inoculated area and these water-soaked spots enlarged during the following 24 hr. The cells within the lesions shrank and the tissues containing the shrunk cells became very thin and yellow halos started to appear around the lesions. Thereafter, the lesion color altered to white or whitish brown and narrow brown bands were sometimes formed around the lesions. The formation process of lesion by the pressure spraying method was very similar to that by the normal spraying method, although the lesion shapes differed from each other. When distilled water was applied to the leaves instead of the bacterial inoculum, no lesions developed.

The observations with a scanning electron microscope showed that the abaxial epidermal cells of healthy cucumber leaves were convex, while stomata were

elleptical in shape (Fig. 1). Similar results were obtained when the inoculated leaves were observed shortly after inoculation. Bacteria were found in some of the stomata within the inoculated areas (Figs. 2a, 2b).

Ingress into the plant tissues by plant pathogenic bacteria occurs through natural openings, wounds and pseudowounds. In this experiment, we used a pressure spraying method with a spray gun instead of normal spraying method. No wounds were produced on the leaf surface by this method. Therefore, it seems that the inoculated bacteria ingress through the stomata. Bacteria which did not ingress after inoculation could be recognized as single or double cells scattered on the leaf surface, as reported by Hass (2).

No marked morphological changes of the epidermal cells were observed within two days after inoculation, but a large number of bacteria were found in the inner parts of the stomata (Fig. 3) and on the surface of the leaves (Fig. 6). Thereafter, the epidermal layer was removed for observation of the spongy parenchyma. Bacteria were found on the surface of the spongy parenchyma cells and some bacterial masses were found in the intercellular spaces (Figs. 4, 5). But the spongy parenchyma cells were not damaged. The bacteria found on the leaf surface seemed to be those that egressed from the stomata after multiplication in the mesophyll. Miles *et al.* (3) also found that bacterial egress from the stomata of peach leaves inoculated with *Xanthomonas pruni* and they thought that these bacteria might be available for new infections.

During the first 3 days after inoculation most of the epidermal cells within the lesions lost their turgor pressure and thereafter became completely flat (Figs. 7–9). As shown in Fig. 10, the lesions were concave and sharply distinguished from the surrounding areas. The epidermal tissues somewhat apart from the lesion either slightly lost their turgor pressure or showed no morphological changes, although bacteria were observed on the surface of those tissues (Fig. 11).

Plenty of small cracks (2–15 μ in length) were formed on the epidermal cells within the older lesion and a large quantity of bacteria were observed in them (Fig. 12). The formation of these cracks on the lesion surface may cause the falling out of the tissues within the lesion which is often observed. The bacteria in the cracks, in addition to those which egressed from the stomata within the lesions at an early stage, also may be useful for secondary infections.

In these experimental conditions, characteristic lesions appeared within the first 4 days after inoculation. From the observations with a scanning electron microscope, the process of lesion formation may consist of 3 steps on the basis of their morphological states: the first step, appearance of water-soaked lesion; the second step, loss of turgor pressure in the cells within the lesion and flattening of the lesion; the third step, cracking of the lesion surface. Presumably the loss of turgor pressure in the lesion might be due to the alteration of permeability in the plasma membranes. Williams and Keen (4), working with cucumber leaves infected

with *P. lachrymans*, have reported that the permeability of infected leaves decreased during the first 24 hr and then increased rapidly, paralleling the increase in size of water-congested lesions. Our view is essentially similar to those of Williams and Keen.

References

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2) Hass, J.H., *3rd ISPP Abs.*, **71** (1978)
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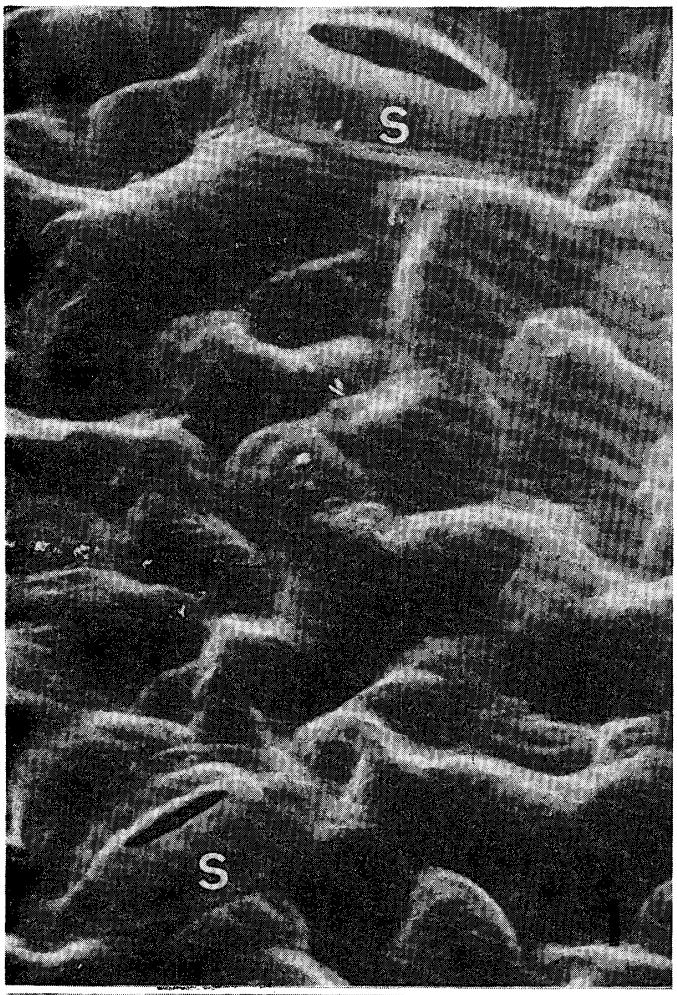
Abbreviations used in Figures

S; stoma, B; bacterium, BM; bacterial mass, SPC; spongy parenchyma cell, L; lesion, SA; surrounding area, LH; leaf hair.

Explanation of Figures

FIG. 1. A surface view of healthy cucumber leaves. ×2000.
FIGS. 2a, 2b. Bacteria found in the stomata within the inoculated areas as observed immediately after inoculation. ×2000, ×4300, respectively
FIG. 3. Bacteria which egressed from the stomata one day after inoculation. ×5100.
FIGS. 4, 5. Bacteria and bacterial masses found in the intercellular spaces of the spongy parenchyma one day after inoculation. The spongy parenchyma cells showed no morphological changes. ×500, ×3000, respectively.
FIG. 6. Bacteria scattered on the leaf surface 2 days after inoculation. ×1000.
FIGS. 7–9. The surface views of the epidermal cells within the lesions at 3, 4 and 8 days after inoculation, respectively. The cells gradually lost their turgor pressure and eventually became completely flat. × 1000.
FIG. 10. The surface view of the lesion 8 days after inoculation. The necrotic lesion was concave and sharply distinguished from the surrounding halo. ×200.
FIG. 11. The surrounding tissue of the lesion 8 days after inoculation. The cells slightly lost turgor pressure or showed no morphological changes. ×500.
FIG. 12. A variety of small cracks produced on the lesion 8 days after inoculation. Bacteria were noted in most of them. ×1600.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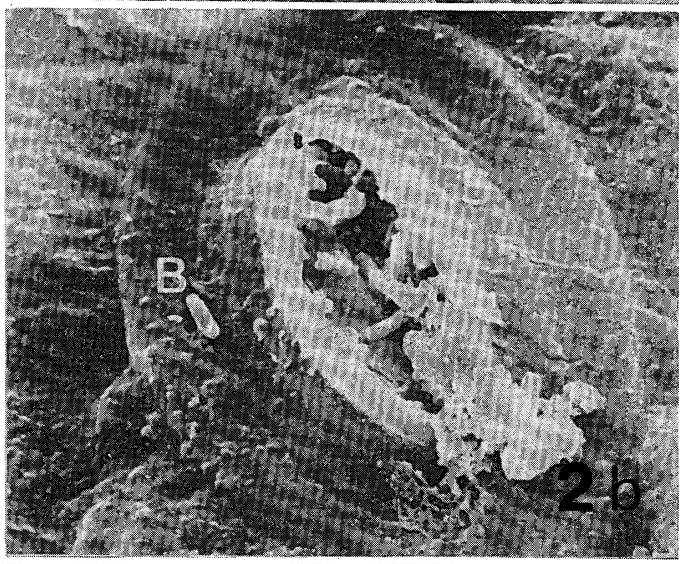
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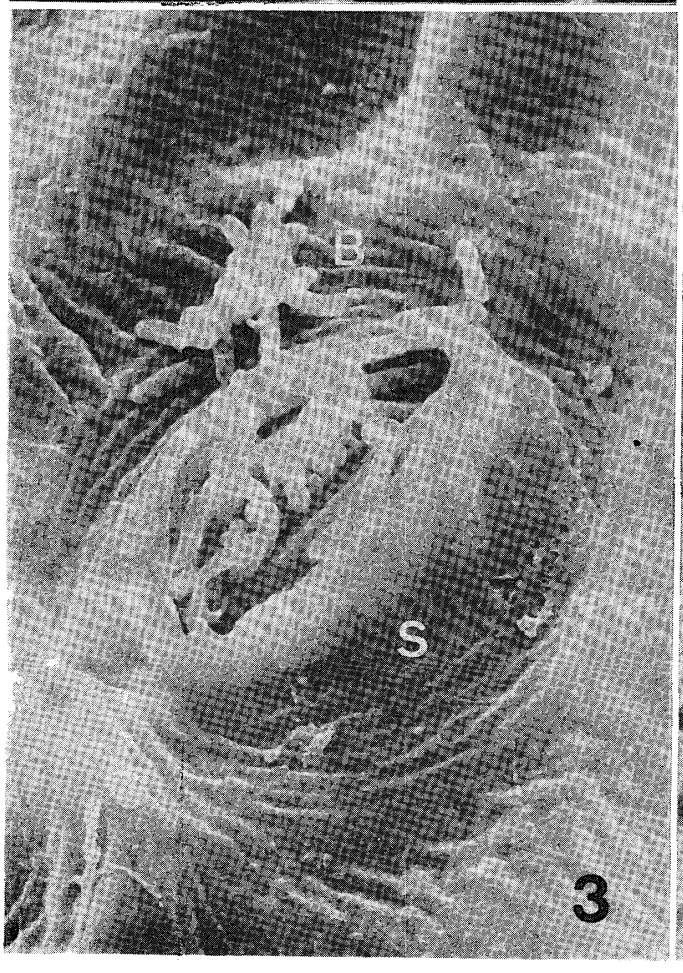
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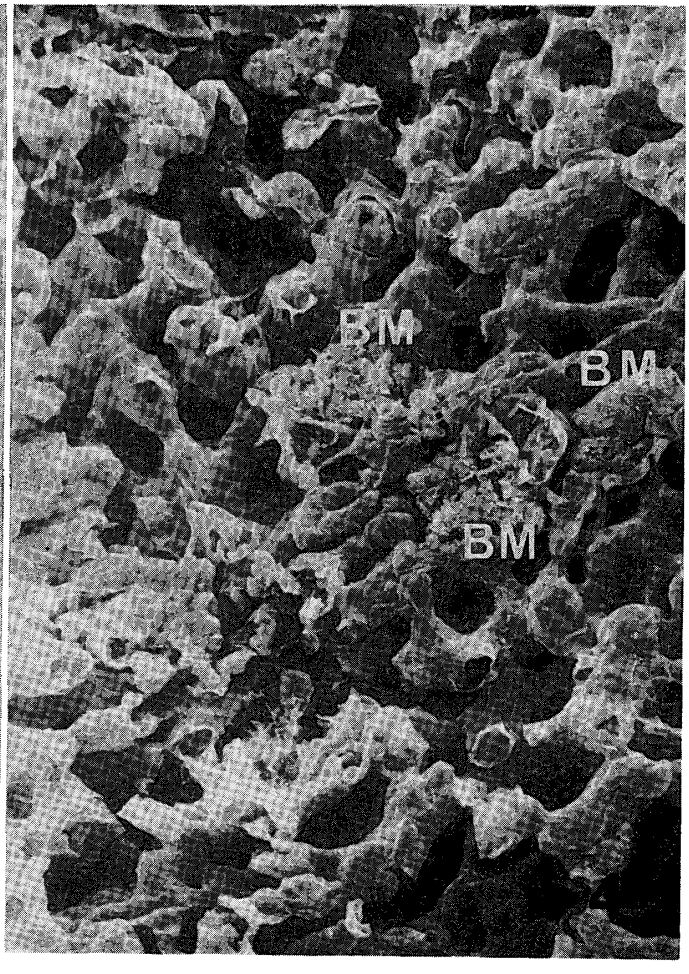
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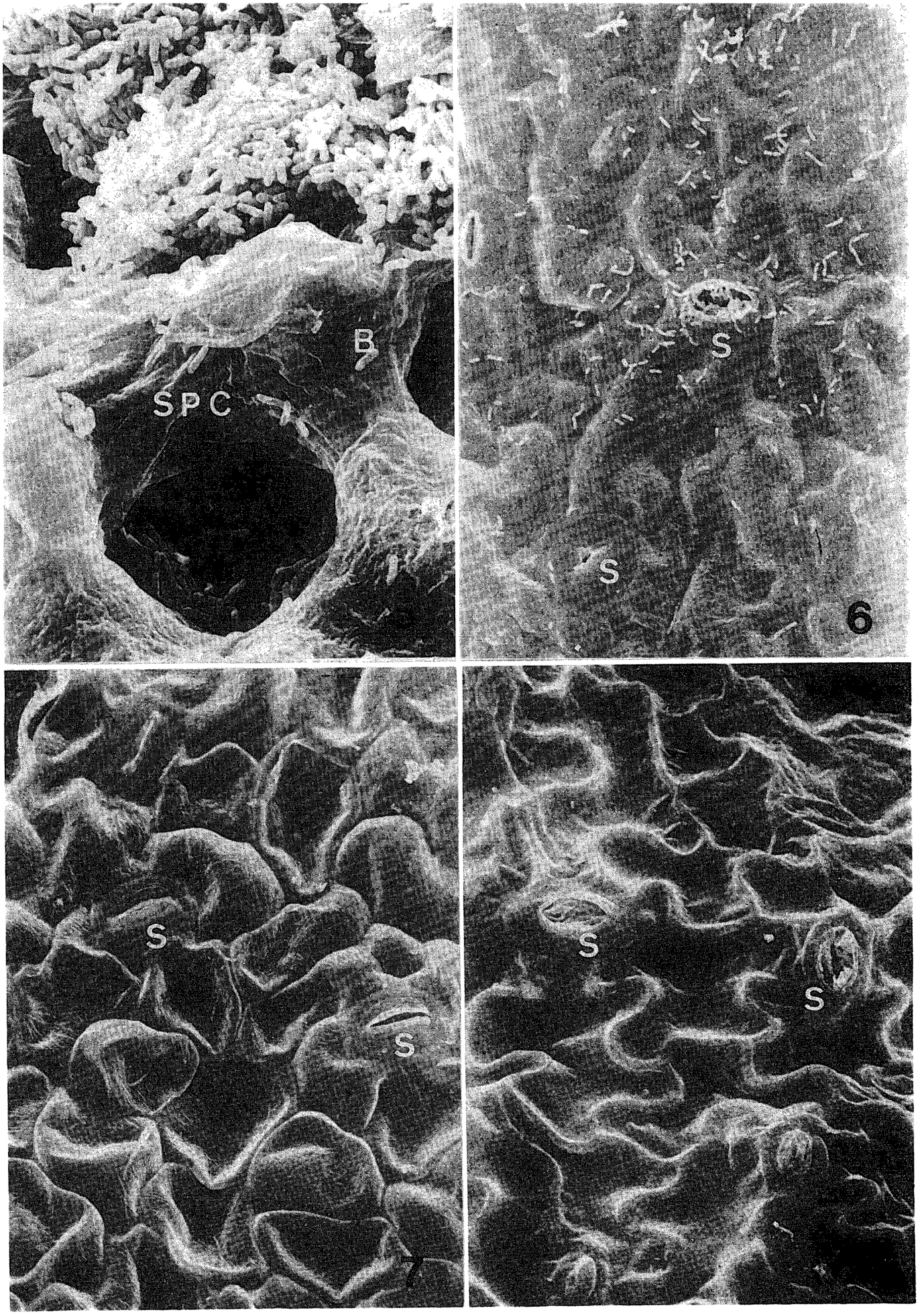
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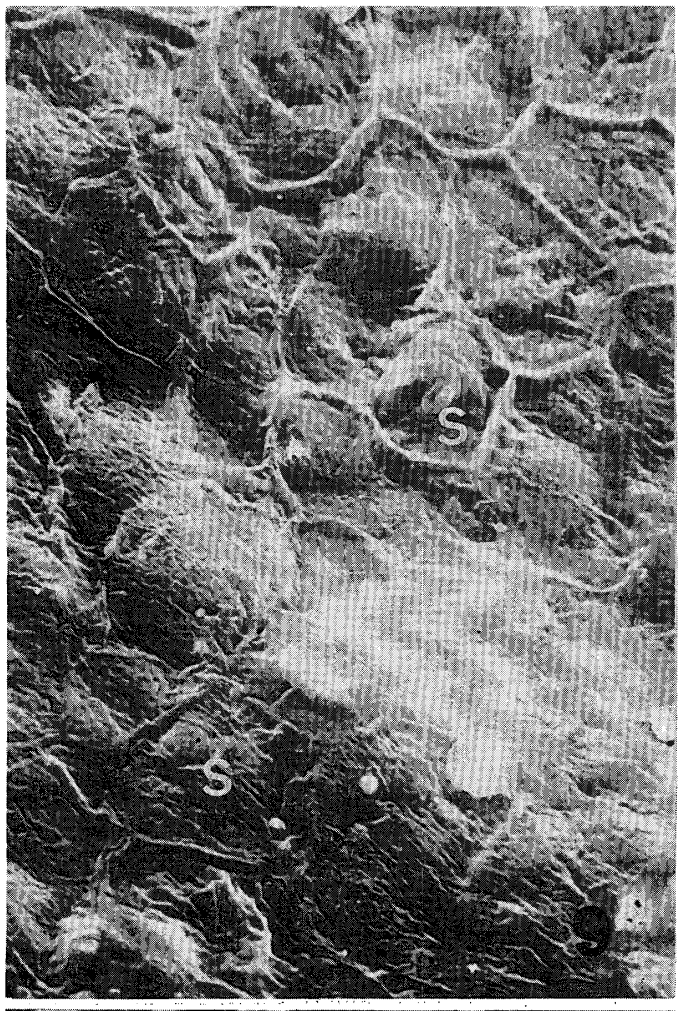
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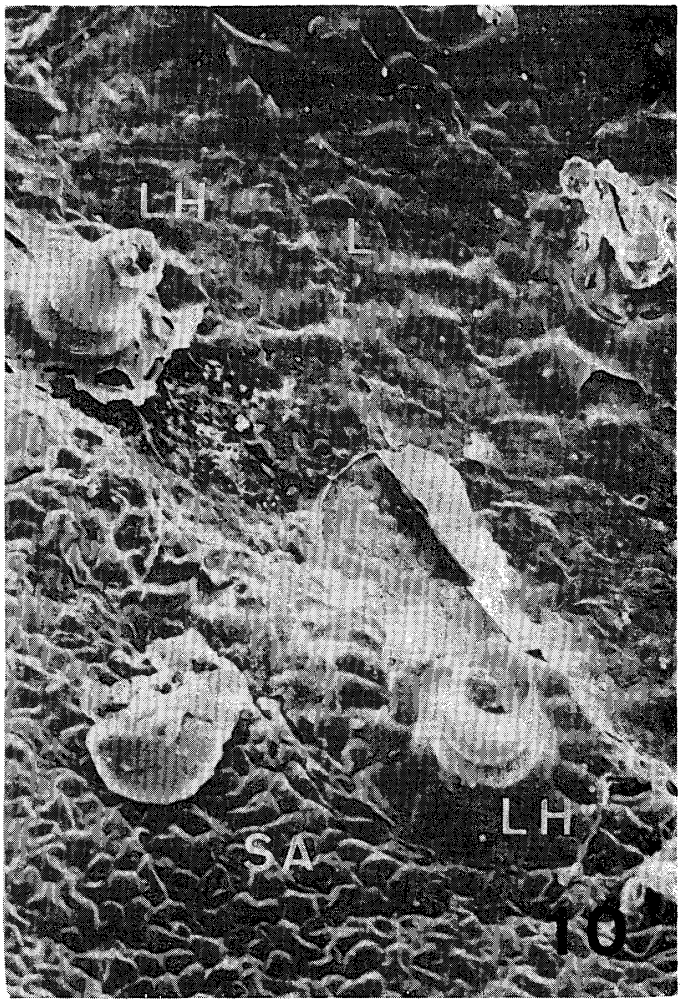
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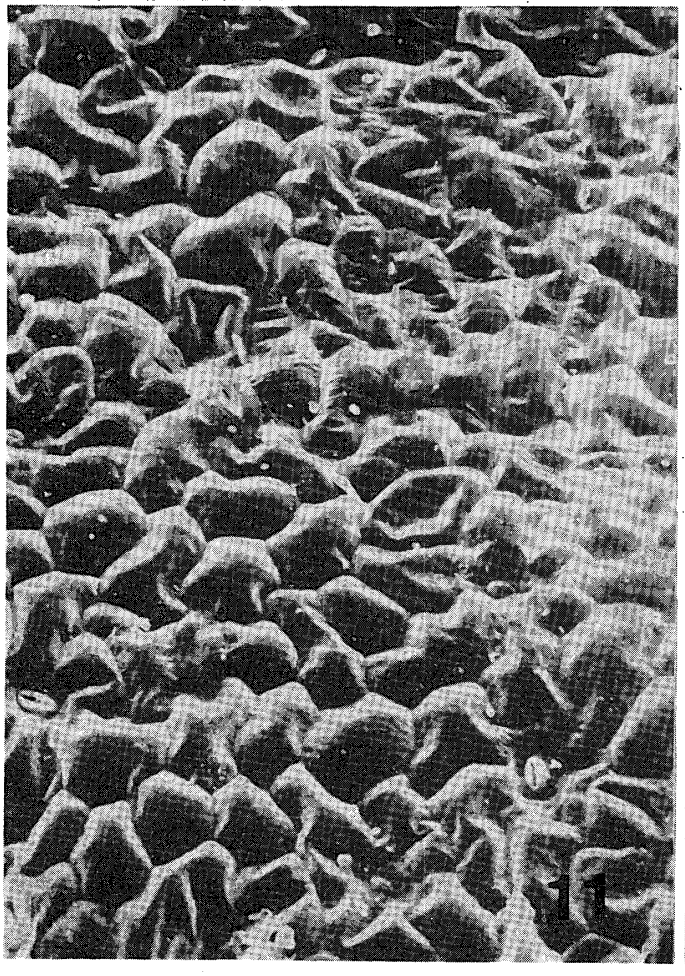
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